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MECHANISMS OF CADMIUM ABSORPTION IN RATS

by

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FOREWORD

The U.S. Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimony to the deterioration of our natural environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The primary mission of the Health Effects Research Laboratory in Cincinnati (HERL) is to provide a sound health effects data base in support of the regulatory activities of the EPA. To this end, HERL conducts a research program to identify, characterize, and quantitate harmful effects of pollutants that may result from exposure to chemical, physical, or biological agents found in the environment. In addition to valuable health information generated by these activities, new research techniques and methods are being developed that contribute to a better understanding of human biochemical and physiological functions, and how these functions are altered by low-level insults.

Cadmium is a highly toxic heavy metal, to which man is becoming increasingly exposed. This report discusses the mechanisms of intestinal absorption of cadmium, and the factors which reduce the net fractional absorption to only a few percent of the oral load, in the hope that the information can be used to reduce cadmium's toxic effect.

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Acting Director
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ABSTRACT

This study was undertaken in order to help clarify the factors which determine the fractional absorption of an oral load of cadmium from the intestine of the rat. The experiments utilized intact segments of intestine, perfused or incubated in situ with their blood supply intact. Absorption of Cd from the jejunal lumen can be ascribed to a saturable membrane system; after short periods of exposure essentially all the metal removed from the lumen is recovered in mucosal tissue. The second step in Cd absorption, i.e. transfer of the metal from mucosa into blood, proceeds at only 1-2% of the rate of uptake from the lumen (Step I). No evidence could be obtained for a role of metallothionein in the mucosal retention of Cd. Step I of Cd absorption is inhibited by a variety of exogenous and endogenous factors. Thus, Zn was found to depress Cd transport in an apparently competitive manner. Addition of milk to the lumen also inhibits Cd uptake, an effect entirely due to its Ca content. Bile salts act as endogenous modulators of Cd absorption; their effect may be related to micelle formation. The work also included studies of duodenal and ileal Cd transport. Ileal Cd absorption differs from that in jejunum by a relatively much faster Step II. Unlike the low ratio of Steps I/II for the toxic metal in the jejunum, that for the essential metals Cu and Zn is much higher (~50%). Absorption of Cd by the gut in neonatal rats proceeds much faster than in adults; reasons for this difference have not yet been clarified. Another question remaining under study is the extent to which different metals such as Cd and Zn share common absorptive mechanisms.

This report was submitted in fulfillment of Grant R805840, awarded by the U.S. Environmental Protection Agency to Dr. E.C. Foulkes in the Department of Environmental Health at the University of Cincinnati. The work was carried out during the period of June, 1978 to April, 1981.

CONTENTS

Foreword iii

Abstract iv

Figures vii

Tables vii

1. Introduction 1

2. Conclusions 2

3. Results and Discussion 4

a) Activity gradients along jejunum 4

b) Kinetic studies of Cd transport out of the lumen 4

c) Interaction between Cd and Zn 5

d) Action of bile salts 8

e) Steps I and II of metal absorption 8

f) Role of metallothionein 12

g) Specificity of metal absorption systems . . . 12

h) Cd absorption in duodenum and ileum 12

i) Cd absorption in the newborn 14

Appendices

		Page
Paper A.	Foulkes, E.C. Some determinants of intestinal cadmium transport in the rat. J. Environ. Path. and Toxicol. 3:471-481, 1980.	17
Paper B.	Kello, D., Sugawara, N., Voner, C. and Foulkes, E.C. On the role of metallothionein in cadmium absorption by rat jejunum in situ. Toxicology, 14:199-208, 1979.	28
Paper C.	Sugawara, N. and Foulkes, E.C. Relationship between Cd and Zn absorption by rat jejunum. Submitted for publication.	38
Abstract A.	Foulkes, E.C. and Voner, C. Steps in Cd transport by rat jejunum in situ. Fed. Proc. 39:1183, 1980	46
Abstract B.	Bonewitz, R.F. and Foulkes, E.C. Modification of jejunal Zn and Cd transport in rats by glucocorticoid not correlated with metallothionein synthesis. Soc. Toxicol. Meeting, San Diego, 1981	47
Abstract C.	Voner, C. and Foulkes, E.C. Inhibition of jejunal Cd absorption in the rat by bile salts. Fed. Proc. 40:1071, 1981. . .	48
Abstract D.	Johnson, D.R., Foulkes, E.C. and Leon, L. Intestinal transport of Cd in newborn rats. Fed. Proc. 40:1073, 1981 . . .	49
Abstract E.	Bonewitz, R.F., Voner, C. and Foulkes, E.C. Comparison of mucosal uptake and transmural transport of Zn, Cu, and Cd in rat jejunum. Fed. Proc. 40:1395, 1981.	50

FIGURES

Number		Page
1	Effect of Zn on Cd uptake	6
2	Effect of Cd on Zn uptake	7
3	Reversible inhibition of Cd transport by bile	9
4	Dose-effect relationship between glycocholate and Cd absorption	10
5	Model of Cd absorption.	11
6	Comparison of Cd, Zn and Cu absorption	13

TABLES

I	Ratio of Steps II/I as function of Step I	12
II	Cd transport in ileum	14
III	Cadmium transport in duodenum of newborn rat	15
IV	Body weight and tissue iron levels	16
V	Iron supplementation and Cd transport in jejunum.	16

SECTION 1

INTRODUCTION

The human environment contains a variety of heavy metals, originating from both natural and anthropogenic sources. Some of these metals are clearly essential for life, while for some others a biological function has been suggested. However, many of these elements, in the form of organic or inorganic compounds, are highly toxic. The level of such compounds in the environment may therefore bear directly on human health. Analysis of their effects assumes additional urgency because of the likelihood that ambient concentrations of these metals may be increasing.

A special problem in this regard is posed by cadmium. Significant amounts of this element are being added to the environment through use of sewage sludges and other fertilizers on agricultural land, from combustion of fossil fuels, and by other processes. The problem is exacerbated by the fact that the half life of Cd in the body, and especially the kidneys, is very long; in effect, Cd acts as cumulative poison. Its prime target organ is the kidney, and significant nephropathy has frequently been reported in exposed human populations.

Main sources of the human body burden of Cd outside of occupational environments are Cd in food, water and tobacco smoke. The non-smoker derives most of his Cd through gastrointestinal absorption. In spite of this fact, relatively little is known about mechanisms of intestinal Cd absorption, and about the factors which on the whole reduce net fractional absorption to only a few percent of the oral load. This consideration defined the objective of research work described in this report. A scientific interest in the basic mechanism of metal transport, and the possibility of applying knowledge gained to the control of metal absorption, formed the starting points for the present investigation.

Throughout the work attempts were made to follow absorption under as physiological conditions as possible, using isolated segments of intestine *in situ* in the living animal. Results obtained are described under appropriate subheadings, but no attempt is made to repeat in detail material already published. Copies of papers and abstracts based on these results are appended to this report. These papers should be consulted for details.

SECTION 2

CONCLUSIONS

The results reported here extend earlier work by other investigators. However, unlike several earlier investigators, our findings were made with intact intestinal segments *in situ* at Cd concentrations one might conceivably encounter in heavily polluted areas. Previous work in many cases had used excessively high Cd concentrations; in addition, the preferred techniques often were the analysis of absorption in the intact animal, or the measurement of transport by everted sacs of intestine *in vitro*. In both cases, the avid retention of Cd in the intestinal wall is a source of difficulty.

Thus, in sacs, cadmium is not likely to diffuse across the submucosal tissues into serosal fluid as readily as, for instance, sugars or amino acids. Purely *a priori*, therefore, the release of Cd from mucosa in sacs may differ quantitatively from that occurring under physiological conditions. In the intact animal the additional difficulty arises that enterohepatic recirculation makes it impossible to obtain absolute values for the unidirectional movement of Cd from lumen into mucosa and blood. We submit therefore the method employed in the present experiments as a more appropriate procedure for the detailed analysis of Cd absorption under reasonably physiological conditions.

If we may accept then the results obtained as approximately reflecting the normal process of Cd absorption the following main conclusions may be drawn:

1) Cd is removed from the lumen of the rat jejunum by a membrane-related process which exhibits saturation kinetics (Step I). After short periods, essentially all Cd thus removed can be recovered from the mucosa. An activity gradient exists along the jejunum.

2) Step I of Cd transport is modulated by bile salts as well as by a variety of food constituents.

3) Zinc interacts in an apparently competitive manner with Cd for transport by Step I. Unlike Cd, however, Zn is not appreciably retained in the mucosa; in spite of the competition

for Step I, the transmural movement of Zn and Cd is not mediated by identical mechanisms.

4) Step II in the absorption of Cd, i.e., its movement from mucosa into blood, proceeds at only 1-2% of the rate of Step I. Step II is in series with Step I, and under present conditions determines the rate of Cd absorption into the body.

5) Metallothionein, the low molecular weight protein able to bind 7 moles Cd/mole, could not be shown to play any role in the absorption of Cd.

6) Cd absorption in duodenum resembles qualitatively that described for the jejunum; in contrast, Step II of Cd transport is relatively much faster in ileum.

In summary, this investigation has contributed to a better definition of factors which may be responsible for the control of Cd absorption in vivo. In addition, it has confirmed the possibility of altering fractional absorption of an oral load of Cd by dietary manipulations. Work along these lines is continuing.

SECTION 3

RESULTS AND DISCUSSION

a) Activity gradients along jejunum: Preliminary studies, in which dilute solutions of $^{109}CdCl_2$ in saline were placed into, or perfused through segments of intestine, had confirmed that isotope is readily removed from the lumen of various segments of the small intestine. Because of its relatively short length, and in order to avoid complications arising from bile secretion (see below), duodenum proved less convenient than jejunum for further detailed studies. Some experiments were carried out with ileal segments but their activity in general was somewhat lower than that of jejunum. Most of the studies reported here were therefore based on jejunum. The proximal portion only was used because of the strong activity gradient along this tissue, with maximum Cd uptake occurring in the first 12 cm distal to the ligament of Treitz (see Figure 1, paper A). Note that in these studies the activity gradient revealed by measurement of Cd disappearance from the lumen agreed closely with the accumulation of the metal in the intestinal wall. This fact will be further considered in section e).

b) Kinetic studies of Cd transport out of the lumen: Because of the tissue heterogeneity, and in order to permit collection of accurately timed samples, a perfusion technique was devised in which a small volume (1.6 ml) of solution is recirculated through the jejunum at 0.4-0.8 ml/min. Details of this technique are described in paper A. When measured in this manner the disappearance of Cd from the perfusate follows first order kinetics (see Figure 2, paper A). Two further points stand out: 1) The rate of exponential disappearance of Cd follows saturation kinetics; in spite of wide variability between animals some approximate values for maximal velocity and affinity constant could be obtained (see Fig. 4, paper A). It is important to emphasize that the reaction studied is only that of accumulation of Cd in the intestinal wall, (Step I of Cd transport), not its absorption into the body (Step II, see sections a and e). It could be further shown that the saturation is at least partially reversible (Table 2, paper A). The apparent saturating effects of relatively high Cd concentrations (0.2 mM) in the lumen cannot be ascribed to general toxic effects of the metal, as volume and glucose uptake remained within normal limits. 2) Step I of Cd transport can be inhibited by Ca. It is

interesting to note that addition of milk to the lumen inhibits Cd transport, and that this effect is fully accounted for by the Ca content of the milk (Table 1, paper A). This finding emphasizes the well-known fact that the composition of the luminal fluid strongly influences metal absorption, i.e. that Cd from a food digest or in presence of normal constituents of luminal fluids is not likely to be absorbed at the same rate as seen here in absence of organic ligands or transport inhibitors (competitors?, see sections c and d).

The finding that Cd and other metals such as Zn are readily removed from the glucose-saline perfusate leads to a further conclusion. Thus, it has been claimed that metals are absorbed as compounds of biological chelators (see e.g. Evans, G.W., Nutr. Rev. 38:137-141, 1980). Under the conditions of the present experiments, however, metal was equally readily absorbed from perfusate free of exogenous ligands, whether this perfusate was recirculated through the intestine so that endogenous chelators might have accumulated, or whether single-pass perfusions were employed. It is clear, therefore, that absorption can proceed without the necessary involvement of endogenous chelators in the lumen. In presence of food constituents etc. the postulated chelators might, of course, contribute to absorption, in competition with non-absorbable metal-ligand complexes.

c) Interaction between Cd and Zn: An interaction between Cd and Zn, possibly competitive in nature, has often been reported and is not surprising in light of the chemical similarities between the two metals. We tested therefore the ability of Zn and Cd to interact at the level of Step I of their transport out of the lumen. Details of these experiments are described in manuscript C. In particular, a double inverse plot (Fig. 1, manuscript C), suggests that the Zn inhibition of Cd uptake may be competitive in nature; similarly, Zn absorption is inhibited by Cd in what also appears to be a competitive manner (Fig. 2, manuscript C). The inhibitory effect of Zn on Cd uptake, and that of Cd on Zn uptake, are also illustrated in Figures 1 and 2 below. The interpretation of these results is somewhat obscured by the fact that Cd appears to have a higher affinity for the transport system than does Zn in Figure 1, whereas the inverse appears true in Figure 2. If, nevertheless, the two metals are competing for a common site then they share at least in part, a common absorption mechanism. At the same time, the overall transport into the blood is different for Zn and Cd. This conclusion is based on the contrast between Steps II for Zn and for Cd: unlike Cd, Zn is retained in the jejunal mucosa to a relatively small extent; the significance of this fact is further considered in section e). A practical implication of the finding of interaction between Cd and Zn is that addition of Zn, as that of Ca (see section b), might conceivably be used to depress the extent of Cd absorption from contaminated food.

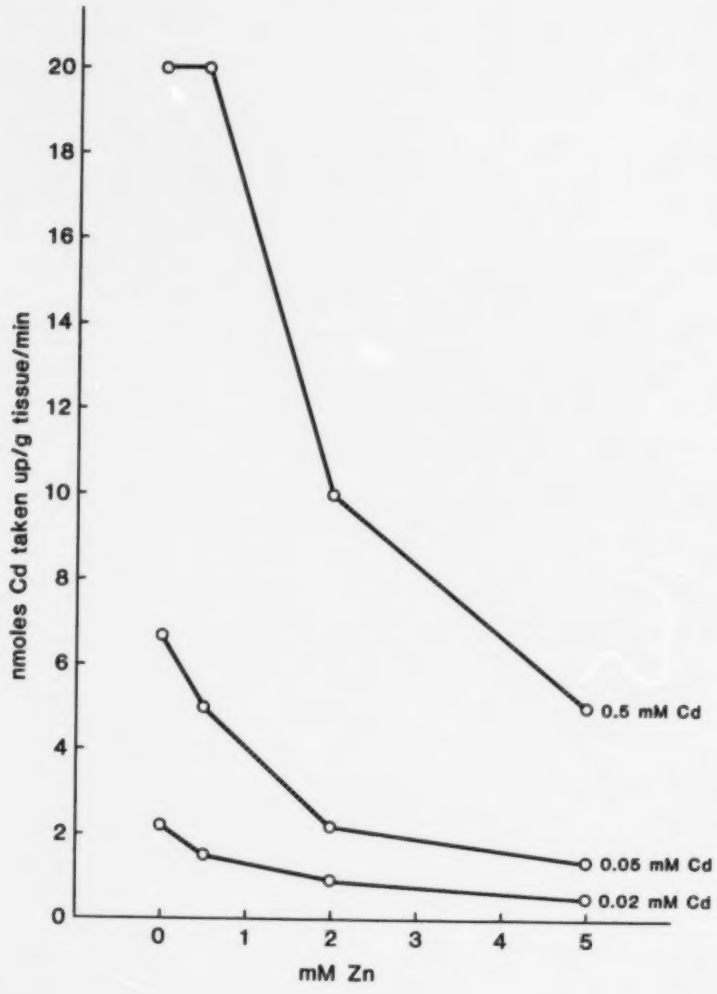

Figure 1

Effect of Zn on Cd uptake

Direct plot of the data from Figure 1, paper C.

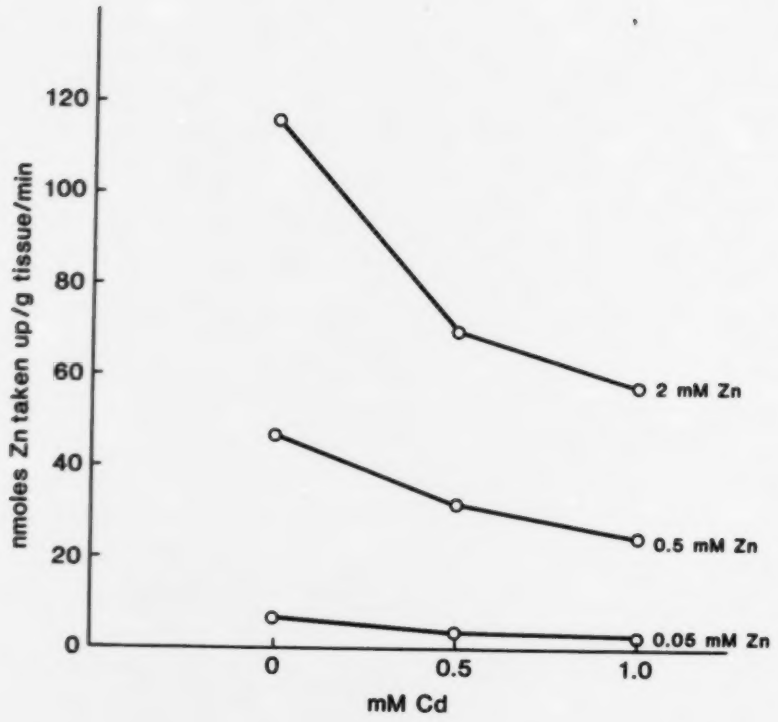

Figure 2

Effect of Cd on Zn uptake

Direct plot of the data from Figure 2, paper C.

d) Action of bile salts: As pointed out in section b) uptake of Cd out of the lumen of the intestine is sensitive to the action of metal ligands and transport inhibitors. Among such compounds are possible endogenous modulators of Cd absorption. Thus, we observed that fresh rat bile contained inhibitor(s) of Step I of Cd absorption, and further work showed this to be due to the presence of bile salts (see abstract C). The effect is freely reversible, as illustrated in Figure 3.

Figure 4 shows a dose-effect relationship for the action of glycocholate on Cd absorption. A close relationship between the critical micellar concentration of the bile salt, and its inhibitory concentrations is apparent. The present hypothesis is that Cd is bound to micelles and thus rendered unavailable for further transport. Preliminary experiments have further indicated that these micelles also interfere with Cd uptake in the ileum. Further work on this question is continuing, but in any case endogenous factors clearly can strongly influence the absorption of Cd. This represents an important conclusion in attempts to explain the low fractional Cd absorption in the intact adult animal.

e) Steps I and II of metal absorption: The results presented so far refer mostly to the removal of Cd (and other metals) from a perfusion solution in the jejunal lumen. As pointed out in section a) there was found close agreement between the rate of this removal and the accumulation of Cd in the tissue. Further work showed that essentially all the Cd thus transported could be recovered in the mucosa. These findings are detailed in paper B. Thus, Table II, paper B shows that after 5 minutes' perfusion, 25.1 nmol Cd had disappeared from the perfusate; 24.5 nmol were recovered from mucosal scrapings. Clearly, therefore, analysis of luminal Cd concentration can yield information only on Step I in Cd absorption. The second step in this process, the release of retained Cd from the mucosa and its further movement into the body, determines the overall rate of Cd uptake into the body.

Determination of Step II of Cd is difficult (see also section g). This fact is due primarily to the small fraction of Cd appearing in blood and tissues. Thus, while it is perfectly feasible to measure Step II of Zn transport in our preparation by serial assays on portal blood, this technique cannot be applied to study of Cd absorption. A new method was therefore devised (see paper B, abstract A): It is based on the fact that, as pointed out above, essentially all Cd transported out of the lumen can be recovered from the intestinal mucosa immediately after the end of perfusion. It is only at significantly later times that some of the mucosal Cd is seen to have moved further into the body. This is illustrated in Figure 1, paper B. The difference between the Cd removed from the intestinal lumen and that recovered from the intestinal wall, e.g. 5 hours later,

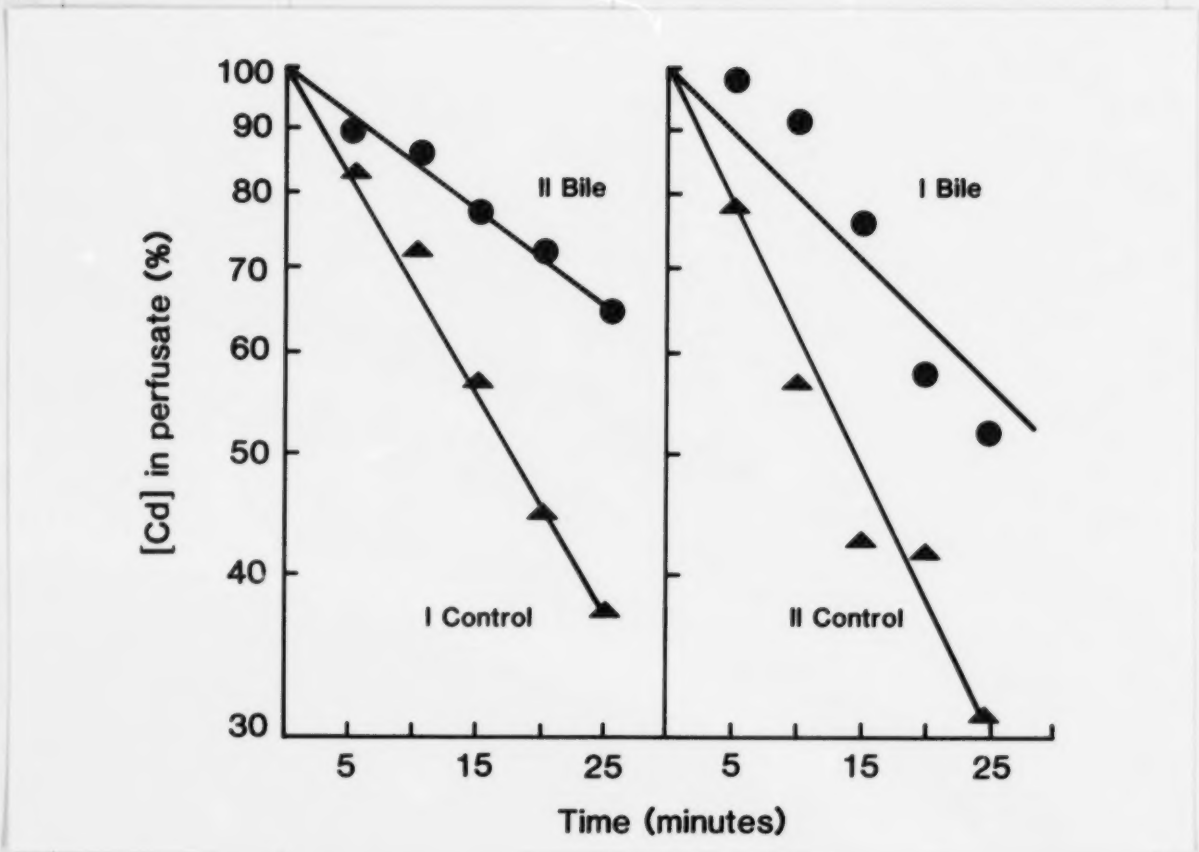

Figure 3

Reversible inhibition of Cd transport by bile

Fresh rat bile (20% v/v) was added to perfusate in period II in left panel, and period I in right panel.

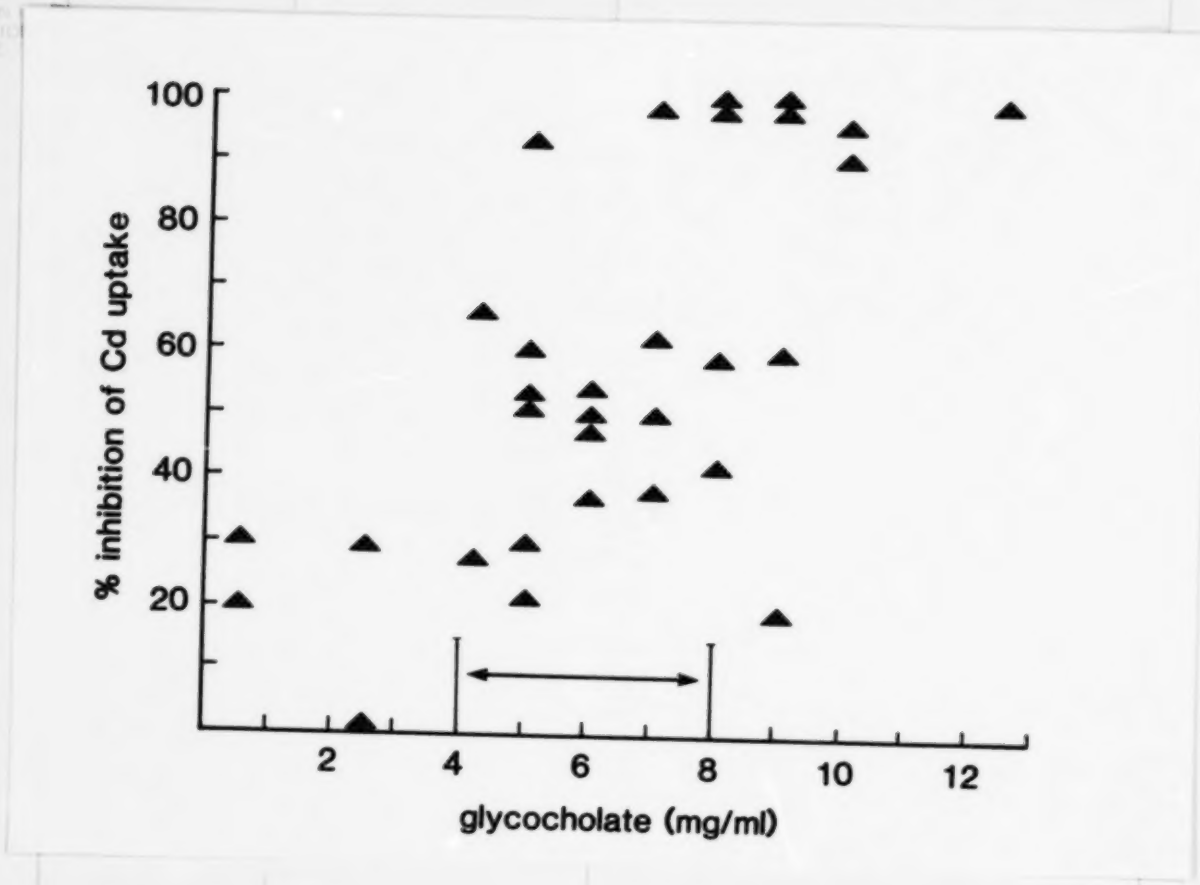

Figure 4

Dose-Effect relationship between glycocholate and Cd absorption

Each point represents the activity in one animal used as its own control. The arrow between 4-8 mg/ml indicates the critical micellar range of glycocholate.

provides a measure of Step II.

A necessary conclusion following upon this kinetic analysis is that Steps I and II represent two processes in series, and that no significant parallel absorption pathway, such as a paracellular shunt, is operating under present conditions. The overall process of Cd absorption under our experimental conditions can then be represented schematically as shown in Fig. 5. Because no significant backflux from mucosa into lumen could be observed, Step I is represented as a unidirectional process. Similarly, under our conditions, blood levels of Cd are very low, so that Step II also can be represented as a unidirectional process. Step II amounted to only 1-2% of Step I (see Table 4, paper B, and Table 1 of this report). In absence from the lumen of ligands and other absorption modulators, Step II therefore determines the rate of overall Cd absorption.

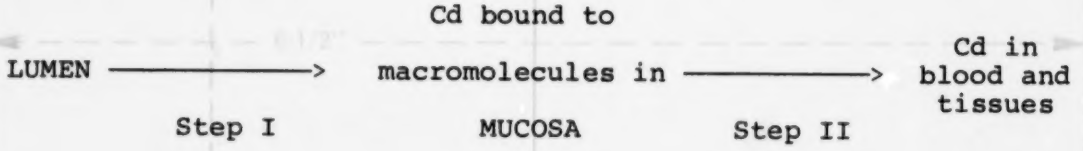

Figure 5

Model of Cd absorption

The model illustrated visualizes a large mucosal accumulation of Cd, presumably due to binding of the metal to various cell constituents. Table 3, paper B, indeed, shows that only about 5% of total Cd taken up by the mucosa is present in low molecular weight fractions. Provided the total binding capacity for Cd in the mucosa has not been exceeded, it then follows from Figure 5 that the rate of overall absorption should be determined by release of Cd from its mucosal ligands. To test this prediction the ratio of Steps II/I was measured over a 10-fold range of luminal Cd concentration, i.e. of absolute amounts of Cd transferred into the mucosa by Step I. This ratio should be independent of the absolute value of Step I. In contrast, one would expect Steps I and II to vary independently if Cd absorption were better represented by a parallel model. Table I summarizes the results of this study, and clearly confirms the prediction of the model shown in Figure 5. Note in this table that over the luminal concentration range studies (20-200 μM), Step I approached saturation; at the same time the ratio of Steps II/I did not change appreciably. Either, therefore, Step II is independent of Step I but exhibits the same saturation kinetics, or more likely, Cd absorption is adequately described by Figure 5.

Table I

Ratio of Steps II/I as function of Step I

Luminal Cd (μM)	n	Step I (nmol/g/min)	Step II / Step I (%)
20	5	2.8 ± 1.6	1.9 ± 1.1
100	6	9.3 ± 4.3	2.2 ± 1.2
200	7	14.7 ± 5.1	2.4 ± 1.9

Results are given as mean ± SD.

f) Role of metallothionein: It has repeatedly been suggested that the low molecular weight metal-binding protein metallothionein (MT) may be involved in the metabolism of heavy metals, and in particular in their absorption. Such a role might consist of increased mucosal metal retention under conditions where presence of heavy metals has previously induced synthesis of MT. This attractive hypothesis was put to the test with Cd. Obviously, the hypothesis predicts that presence of excess MT in the cell would lead to depression of Step II of Cd transport as opposed to Step I. Paper B shows that rats whose Cd binding capacity in the MT fraction of the mucosa had been increased almost 4-fold (Table I, paper B), and who accordingly retained increased amounts of freshly absorbed Cd in their MT (Table III, paper B), showed the same ratio of Steps II/I as did control animals (Table IV, paper B). There is little support here for the suggestion that MT might be involved in the control of Cd absorption.

g) Specificity of metal absorption systems: As mentioned under subheading c), there is evidence that Zn and Cd share at least in part a common mechanism of absorption. Further tests of this hypothesis have been initiated with Zn-deficient rats. In such animals the rate of overall Zn absorption is homeostatically increased. If, now, Zn is transported by the same system as is Cd one would predict that Cd uptake in such animals would also be accelerated. Whatever the outcome of these studies, there are clear differences, however, between the overall processes of Cd and Zn absorption. These experiments have also been extended to the study of Cu absorption, and are illustrated in Figure 6 (see abstract B; paper in preparation).

h) Cd absorption in duodenum and ileum: A complete evaluation of the mechanism of intestinal Cd absorption requires extension of the work so far reported to other sections of the small

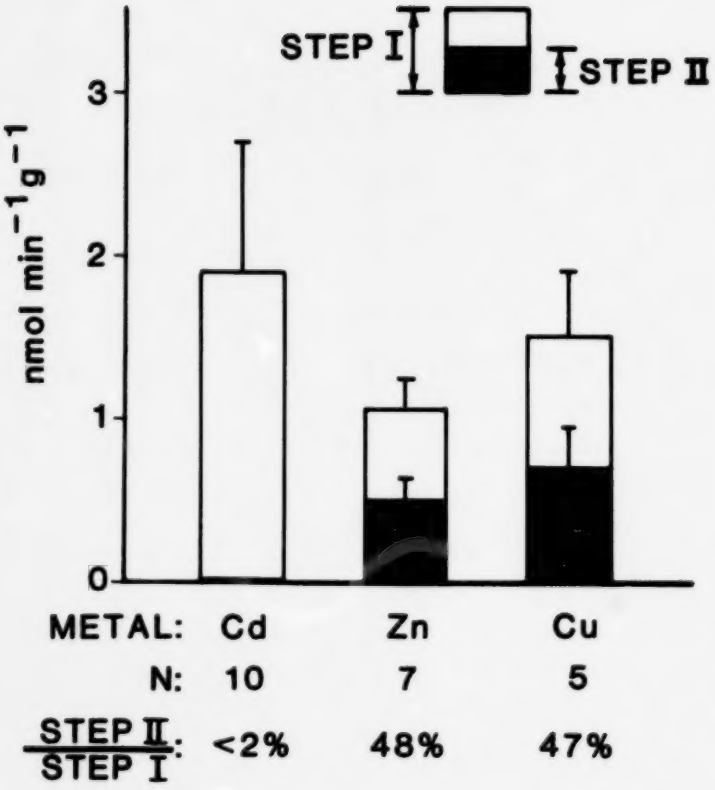

Figure 6

Comparison of Cd, Zn and Cu absorption

intestine. Accordingly, studies were carried out with isolated segments of ileum and duodenum, similar to those employed in the analysis of metal transport in the jejunum. Characteristics of Cd absorption in duodenum resemble those observed in the jejunum, with a Step I transporting Cd out of a 20 μM Cd solution at a rate of 1.8 ± 0.9 nmol/g/min, and Step II being very slow. In such studies it is essential to tie off the common bile duct; otherwise, especially in experiments employing recirculation of the perfusate, the accumulation of bile depresses Cd absorption, as it does in the jejunum.

Ileal Cd absorption, in contrast, differs significantly from the process in jejunum. This fact can be seen in Table II: note first the somewhat lower rate of Step I, and secondly the relatively and absolutely much greater rate of Step II (about 50% of that of Step I). Under suitable conditions a bile salt inhibition of ileal Cd uptake can be demonstrated, but this work is still in progress.

Table II

Cd Transport in Ileum

Animal Number	Step I (nmol/g/min)	Step II/I (%)
1165	1.3	86
1166	1.0	56
1169	1.5	72
1170	0.9	61
1259	0.2	54
1260	1.3	63
1261	0.6	28
1262	1.5	20
	1.0 ± 0.5	55 ± 22

i) Cd absorption in the newborn: This work was begun because of the well-documented observation that Cd, like many other metals, is absorbed more readily in the newborn than in the adult. This raises the following basic questions: a) Is the transport mechanism for Cd in the newborn similar to that in the adult although functioning at a greater rate; alternatively, are one or two distinct mechanisms involved? b) If two distinct mechanisms exist, what factors influence the developmental change in absorption of Cd in the newborn? c) Are Cd and Zn transported

by the same mechanisms at the level of Step I? If so, developmental changes should be the same for Cd and Zn absorption.

Seventeen-day pregnant Sprague-Dawley rats were housed in individual breeding cages. Upon birth of the pups, litters were culled to between 8 and 12 animals each. The majority of experiments were performed between day 12-16 after birth. Rat pups were weighed and anesthetized with Inactin (100 mg/kg body wt, i.p.). The animals were placed on a heated surgical table and the intestine was exposed. Initial attempts to measure Cd transport using the recirculating system used in adult studies were unsuccessful because of lack of an adequate volume marker. Consequently stationary segmental incubation in situ was employed as in section a). Briefly, the duodenum from the pylorus to the ligament of Treitz or a segment of proximal jejunum were isolated. The segments were washed out with saline followed by air; 0.05-0.50 ml 20 μM $CdCl_2$ glucose-saline was then placed into the segment. The segments were tied off and replaced into the abdomen for 30 minutes. The animals were then killed, the remaining intestinal fluid collected, and the lumen washed with 10 mM Na_2 EDTA. The wash and remaining luminal fluid were combined. Fluid and tissue were counted for ^{109}Cd activity. Percent Cd uptake was calculated as 100% - % dose remaining in the intestinal lumen. Percent Cd absorption was calculated as 100% - % dose remaining in lumen + % dose found in intestinal wall.

Results of experiments to determine the effect of time on Cd transport in the neonatal duodenum are shown in Table III. Removal of Cd from the lumen is rapid, but as in the adult, the major portion of the removed Cd can be recovered in the intestinal wall.

Table III

Cadmium Transport in Duodenum of Newborn Rat

Incubation Time	Recovery in Tissue	Recovery in Lumen	Absorbed
min	%	%	%
1	85 ± 4	19 ± 5	0
5	83 ± 6	15 ± 2	2
10	81 ± 9	11 ± 4	8
20	88 ± 4	6 ± 1	6
30	81 ± 4	6 ± 2	13

Values represent mean ± SEM of 3-4 animals, and are expressed as % of original amount of Cd placed into the lumen.

The factors determining the high Cd uptake in the newborn intestine remain unknown. One factor suggested is the low iron concentration in mothers' milk. Iron deficiency, at least in the adult, increases intestinal transport of both Fe and Cd. If Fe status influences intestinal Cd transport similarly in the newborn, correction of the Fe deficiency resulting from low iron intake would reduce Cd uptake. To test this hypothesis, rat pups from day 7 were fed 0.5 ml cow's milk/day, with or without supplementary Fe (200 ppm $FeSO_4$). After one week, Cd absorption was measured as usual and the Fe content of intestine and liver determined by atomic absorption photometry.

Table IV shows intestine and liver iron levels in control and iron-supplemented rat pups. Clearly, there is an increase in iron concentrations in both tissues as a result of iron supplementation. Table V presents data on intestinal Cd transport in newborn rats. No difference between control and Fe supplemented rats could be found in the percent of ^{109}Cd dose removed from the intestinal lumen or retained in the tissue.

The results of these experiments indicate that increased iron levels of intestinal mucosal cells do not reduce Cd uptake in newborn rats. Thus, unlike in the adult, Fe status does not appear to be a critical influence on Cd uptake in the newborn. This finding suggests the possibility that intestinal Cd transport in the newborn differs qualitatively from that in the adult.

Table IV

Body Weight and Tissue Iron Levels

	Body Weight g	Intestine µg Fe/g wet wt.	Liver
Control (5)	35 ± 3	27 ± 4	42 ± 9
Fe Supplemented (5)	35 ± 3	41 ± 6	260 ± 58

The number of animals used in each group is in parentheses. Values are presented as the mean ± SEM.

Table V

Iron Supplementation and Cd transport in Jejunum

	% Remaining in Sac	% Dose in Intestinal Mucosa	% Dose Absorbed
Control (5)	8.4±2.5	77.4±1.4	14.2±1.3
Fe Supplemented (4)	9.4±1.6	79.8±3.9	11.1±3.6

The number of animals used in each group is in parentheses.

Values are presented as the mean ± SEM.

APPENDIX

Paper A

SOME DETERMINANTS OF INTESTINAL CADMIUM TRANSPORT IN THE RAT[1,2]

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The hypothesis was tested that Cd absorption from the intestinal lumen is mediated by cellular transport systems. Cd is readily extracted from glucose-saline during perfusion of jejunal segments in the living rat. Over periods as long as 40 minutes, essentially all extracted Cd is recovered in the wall of the intestine. Cd uptake by the tissue obeys saturation kinetics with K_M values of the order of 0.1 mM, and V_{max} approximately 0.01 μmol/g/min. Although washing after exposure to ^{109}Cd removes only little radioactivity from the tissue, it reverses at least partly the saturating effects of higher Cd concentrations. Unidirectional flux of Cd into the tissue is inhibited by 10 mM Ca; no effect on backflux of Cd is seen. In contrast, Zn and EDTA both accelerate washout of Cd. The Ca content of skimmed milk fully accounts for the depressing effect of dried milk on Cd uptake. These results point to the presence in mucosal cell membranes of a saturable process responsible for Cd uptake and sensitive to inhibition by certain solutes in the lumen.

INTRODUCTION

Outside the occupational environment, ingestion represents the major route of human exposure to Cd. Net fractional absorption of the metal from the gut, however, amounts to only a few percent of the oral load (Moore *et al.*, 1973); the factors restricting absorption to such low values are not well understood. One important variable undoubtedly is diet and the presence in the intestinal lumen of dietary constituents and other compounds which might either directly affect systems involved in Cd transport, or indirectly exert their effect by reacting with Cd, thus altering its diffusibility and transport characteristics. Thus, chronic milk feeding was reported to increase Cd absorption in young rats (Kello and Kostial, 1977). A direct influence of intraluminal

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[2]A preliminary report appeared in the Physiologist, Vol. 21, p. 38, 1978.

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reactions of Cd is seen, for instance, in the work of Kojima and Kiyozumi (1974) and of Cherian *et al.* (1978). Effects of Cd chelators on epithelial Cd uptake have also been noted in the renal tubule (Foulkes, 1974).

Although it has been reported that movement of Cd out of the intestinal lumen obeys first order kinetics (Kojima and Kiyozumi, 1974), results of the present work suggest a different conclusion. Saturation of a hypothetical transport system, then, or its low affinity for Cd, could constitute additional determinants of Cd uptake. A suggestion that proteins similar to metallothionein may be involved in Cd absorption (Evans *et al.*, 1970) is attractive but unproven. Sugawara and Sugawara (1977) proposed a role for metallothionein in the well-documented prolonged retention of Cd in the intestinal wall; sloughing of mucosal cells with their metal content presumably also contributes to the low net Cd absorption into the body (Richards and Cousins, 1974).

Present understanding of reactions involved in intestinal Cd transport does not permit a full evaluation of all these possibilities. The work reported here was undertaken in order to explore further the contribution of various factors to Cd uptake from the lumen of the rat jejunum.

MATERIALS AND METHODS

Male Sprague-Dawley rats, weighing 250-300 g, were maintained on commercial chow (Purina) and tap water *ad lib* for at least one week before study. Twenty-four hours before the experiment, food was removed. Anesthesia was induced with Inactin (100mg/kg IP); body temperature was maintained close to 37°C by means of a rectal probe and a thermostatically-controlled heat pad. The trachea was cannulated. In some studies mean arterial blood pressure was determined in the femoral artery with a Harvard Instrument Co. transducer; in general, blood pressures remained normal throughout the studies. A 15 cm length of jejunum, starting at the ligament of Treitz, was cannulated at both ends and perfused at varying rates from a reservoir kept at 37°C. The problem of mixing and therefore of long periods (>20 minutes) required before effluent concentrations reached steady values interfered with use of physiologically reasonable flow rates (<0.1 ml/min). With a somewhat higher flow (0.4 ml/min, average perfusion pressure 2 cm H_2O) 5 minutes sufficed to attain a steady state which could thereupon be maintained for at least 30 minutes. As many as 4 different solutions could thus be tested sequentially over a period short enough to assure the stability of the preparation. In some studies, perfusion solutions were recirculated through the intestine. Inclusion of the reservoir in the dead-space volume necessitated further increases in perfusion rate. This procedure permits convenient sequential sampling for the determination of transport kinetics. Transit time of perfusate through the intestine was estimated with a small bolus of Dextran Blue in saline, and luminal volume was calculated as the product of perfusion rate and transit time.

Under no conditions did it prove practicable to perfuse more than 2 or 3 segments of intestine simultaneously, making difficult simultaneous comparison of effects of several variables. In one series of experiments, therefore, the

cannulated intestine was filled as usual; 3 cm sections were then tied off and the abdomen closed for 30 minutes (stationary incubation). Mixing of luminal contents in such short isolated segments proved inadequate for sequential sampling. After stationary incubation, therefore, the intestine was removed for analysis of contents and tissue in each segment, as well as for evaluation of Cd uptake by the remaining carcass in a Packard whole body counter.

The standard intestinal solution contained 5 mM glucose in saline, together with 0.1 μCi/ml ^{3}H-polyethylene glycol (New England Nuclear) as volume marker. Glucose was added because of its tendency, observed early in this study, to support constant Cd transport. $CdCl_2$ labelled with ^{109}Cd (0.02 μCi/ml) was added to the desired concentration, and removal of Cd from the lumen was calculated from changes in the ^{109}Cd/^{3}H ratio as determined on a Packard liquid scintillation spectrometer with automatic external standard. Rates are expressed as μmol Cd removed/unit weight or length tissue/minute. Tissues to be analyzed were first cut open, then rinsed for 10 seconds in saline, blotted on the serosal side, and finally weighed and measured. Cadmium content of the tissue was determined on a Packard well-type scintillation spectrometer. Knowledge of the relative counting efficiencies made it possible directly to relate disappearance of Cd from the lumen with its accumulation in the tissue. Glucose absorption from recirculating perfusate was measured with a glucose oxidase procedure.

RESULTS

Activity gradient along jejunum

Ideally one would wish to compare simultaneously effects of several variables on Cd removal from the lumen. To determine the feasibility of such a procedure, changes in the luminal ^{109}Cd/^{3}H ratio were measured in a series of contiguous 3 cm segments after a period of 30 minutes (stationary incubation). Fig. 1 shows results of 1 of 3 such studies.

An activity gradient along the intestine is clearly apparent, with the ability to remove Cd from the lumen mirrored by its accumulation in the tissue. Existence of this gradient makes it impossible to compare simultaneous activities in more than 2 contiguous segments. Study of 2 segments proved useful, provided each comprised about 1/2 of the average length employed for experiments similar to that shown in Fig. 1 and provided the order of addition of control or experimental solution was alternated. On this basis consistent results could be obtained in the study of a single variable, each animal serving as its own control (cf. Table 1).

Extent of Cd transport

The ability of the intestinal wall to accumulate and retain Cd is well documented. Under present conditions also, as seen in Fig. 1, Cd is accumulated in the tissue. In two studies the absolute loss of Cd from the lumen (perfused with 0.02 mM Cd) was calculated from the combined volume of

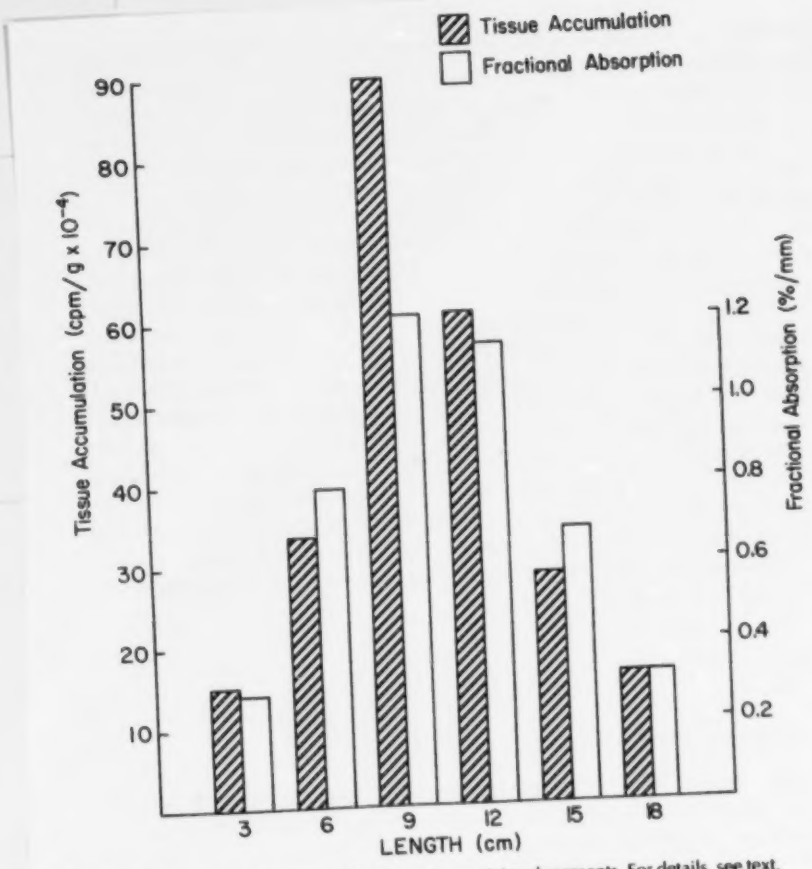

FIGURE 1. Stationary incubation (30 min) in contiguous jejunal segments. For details, see text.

TABLE 1. Effect of Milk on Cd Removal

Solution	n	Fractional Removal[a] (%)	Activity (% of control ± SE)
Saline	15	21 (range 10-48)	100
10 mM $CaCl_2$ in saline	5	12	55 ± 6
4% (w/v) SMP[b] in saline	10	10	50 ± 10

[a]Removal of Cd was measured during 30 minutes' stationary incubation in 2 contiguous segments, each animal serving as its own control; removal was normalized for tissue weight. [b]SMP: skimmed milk powder, containing 0.25 nmol Ca/g, i.e. final Ca concentration 10 mM. Concentration of ^{109}Cd: 0.02 mM.

luminal fluid recovered and saline washings, together with the $^{109}Cd/^{3}H$ ratio. Tissue recovery of Cd lost from the lumen amounted to 85 percent (experiment 1, 12 minute perfusion) and 79 percent (experiment 2, 40 minutes). In two further studies on 2 animals each, 1 ml of the usual 0.02 mM Cd solution was introduced into the jejunum. One animal in each pair was killed immediately and the gut removed; stationary incubation in the second animal continued for 30 minutes. As counted with the whole body counter, the ratio of carcass activity in animal 2/animal 1 never exceeded 1.0. In other words, no significant transmural movement of Cd occurred under present conditions.

Effects of milk

The stationary incubation technique described above was used to determine effects of some soluble dietary constituents (milk) on Cd removal from the lumen. Each segment contained 1 ml saline (glucose was omitted in these experiments); dried skimmed milk powder (40 mg/ml) was added randomly to one of the 2 contiguous segments under study. Table 1 illustrates the strong inhibition exerted by milk constituents. In each case, activity in the control segment simultaneously determined was equated to 100 percent. Fractionation of the milk powder showed no activity in the protein fraction isolated on Sephadex G75. Instead, full inhibitory power was retained upon wet ashing of the powder. Finally, as also shown in Table 1, the Ca content of the ash adequately accounted for the ability of milk acutely to interfere with Cd removal.

Ca inhibition of Cd flux

More detailed understanding of Cd translocation and its inhibition by Ca requires information on kinetics of the process. Fig. 2 illustrates the rapid removal of Cd from a recirculating perfusate; also shown are the Ca inhibition previously observed during stationary incubation and the saturating effect of high Cd concentration.

Ca inhibition of net Cd removal out of the lumen involves depression of flux from lumen to tissue, not accelerated washout of Cd from the tissue. This is illustrated in Fig. 3 by results of one of 3 similar studies in which intestines had been preloaded by perfusion for 20 minutes with 0.02 mM ^{109}Cd in saline-glucose as usual. The perfusate was then replaced with a Cd-free solution, and the ratio of residual ^{109}Cd to ^{3}H was equated to 100 percent. During the next 15 minutes only little ^{109}Cd was washed out of the tissue, as deduced from the slow rise in the isotope ratio. This control washout rate was not altered by addition of 10 mM Ca. The inactivity of Ca may be contrasted with the effects of Zn and EDTA, as determined in separate studies; both substances strongly accelerated washout. Zn not only accelerates washout of Cd, but also depresses the forward flux of Cd, as shown in experiments (not further detailed here) in which initial Cd removal from the lumen was measured in intestines not previously exposed to Cd.

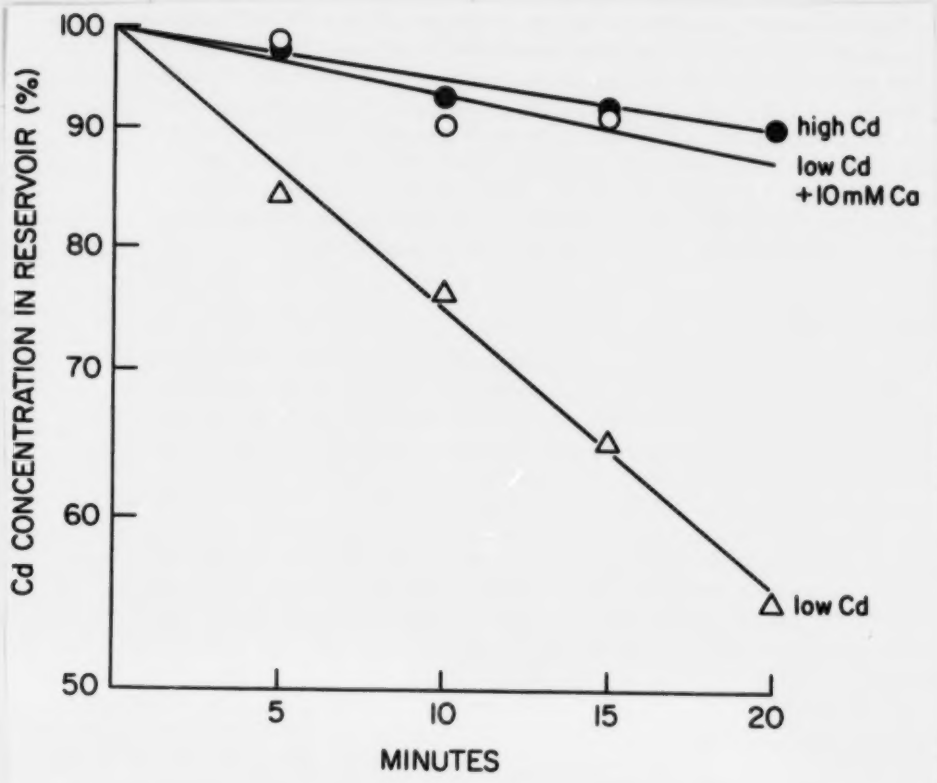

FIGURE 2. Time course of Cd uptake from lumen of perfused jejunum. Initial concentrations of Cd were 0.02 mM and 0.20 mM; total volume perfusate 3 ml, perfusion rate 0.6 ml/min, length tissue 22 cm.

Kinetics of Cd removal

Although reduced fractional Cd removal at higher Cd concentrations could be readily demonstrated in recirculation experiments (Fig. 2), somewhat more consistent results were obtained with steady state perfusion at 0.4 ml/min. Even with this technique, however, great individual variation was found, as shown in Fig. 4 by the results of three consecutive studies. Similar results were repeatedly obtained (Table 2). Although these further experiments all confirmed the saturability of Cd transport, only two Cd concentrations were studied in each, and they have, therefore, not been included in Fig. 4. What is very apparent in both Fig. 4 and Table 1 is the great variability between animals; this renders difficult attempts to define accurately the kinetic constants of Cd transport.

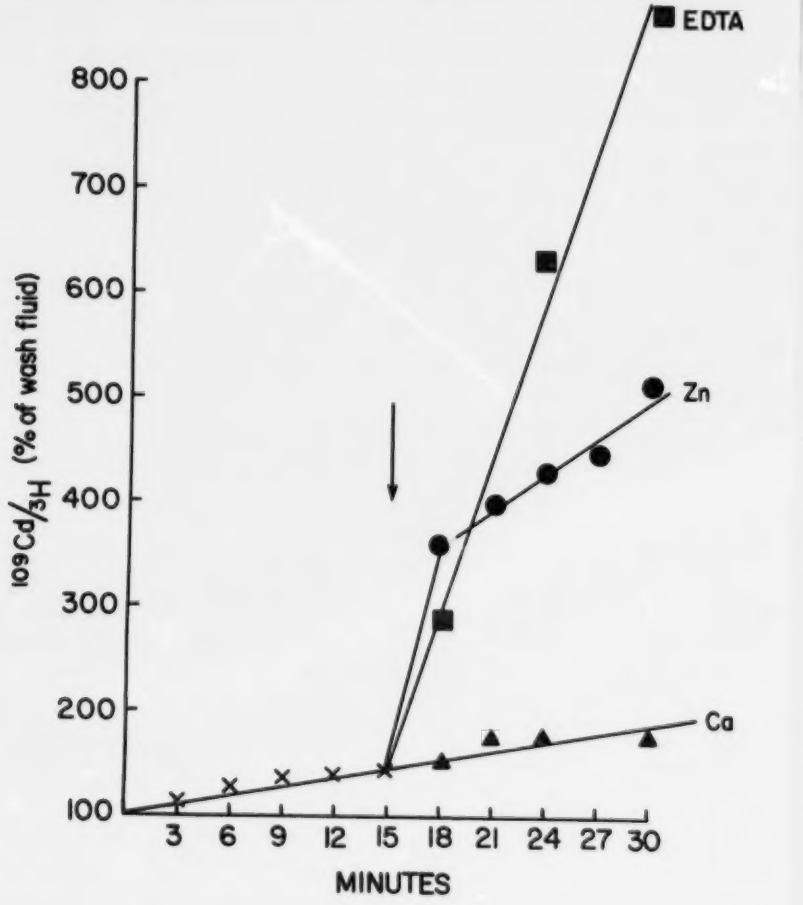

FIGURE 3. Reversal of Cd uptake from lumen. Tissue was preloaded with ^{109}Cd as described in text; perfusate was then diluted 20-fold with Cd-free solution, and the ratio of residual ^{109}Cd to ^{3}H equated to 100. After 15 minutes 10 mM Ca, Zn or 5 mM EDTA were added as shown.

To test the hypothesis that the effect of high Cd concentrations represents a non-specific intoxication of the system, two experimental approaches were used. In two studies removal of glucose from the perfusate was followed over a period of 25 minutes and found not to be reduced by 0.5 mM Cd. In another two animals, jejunum (28 cm) was filled with glucose-saline as usual. The outflow cannula was closed and the lumen connected to a horizontal pipette

whose emptying provided a direct measure of volume absorption. Control values of fluid absorption were 0.17 and 0.27 ml/minute compared to 0.21 and 0.25 ml/min after addition of 0.5 mM Cd. In other words, no acute effects of 0.5 mM Cd on intestinal function could be observed. Substitution of 5 percent mannitol for the saline completely abolished volume absorption and provided a positive control.

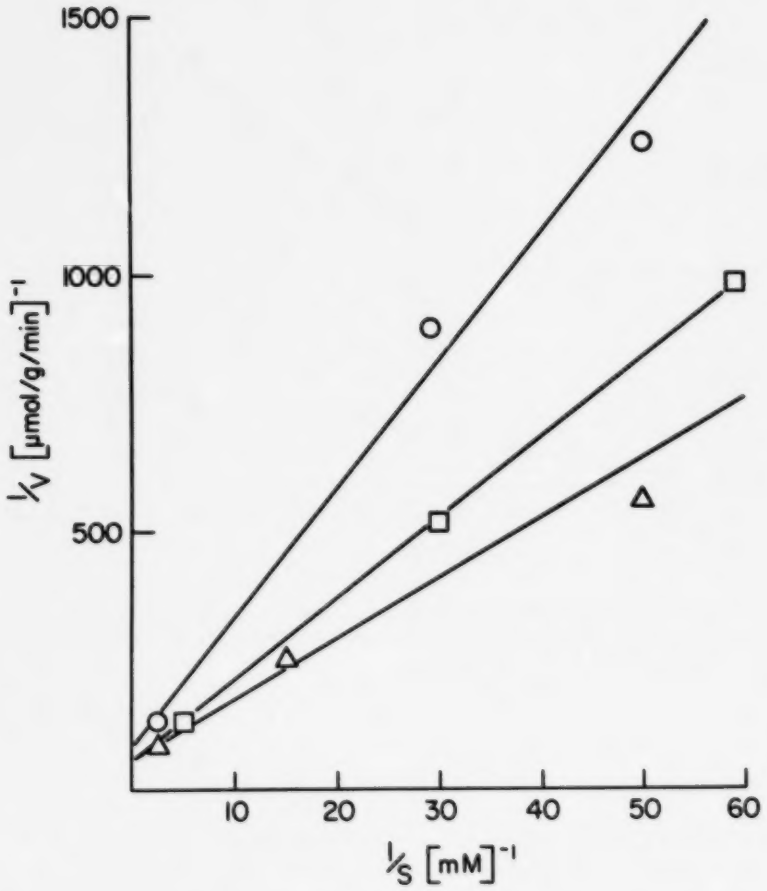

FIGURE 4. Kinetics of Cd absorption from lumen. Steady-state perfusion at 0.4 ml/min; results of 3 different animals are shown.

TABLE 2. Reversible Saturation of Cd Transport

Experiment #	Cadmium Transport					
	Period I (.02 mM)		Period II (nM)		Period III (.02 mM)	
	A	B	A	B	A	B
1	3.5	100	0	0	1.2	34
2	1.8	100	9.6	53	1.3	72
3	2.2	100	4.0	18	2.0	91
4	5.5	100	17.6	32	1.9	35
5	3.4	100	4.0	12	1.1	32
5	2.9	100	0	0	1.1	38
Mean		100		19		50

Perfusion rate 0.4 m1/min. Cd transport is expressed in Column A in nmol/g/min, in B as fractional absorption in percent of control. The initial Cd concentration for each period is also shown.

Reversibility of saturation effects

In nine experiments, jejunum was first perfused as usual at 0.4 ml/min with glucose-saline containing 0.02 mM ^{109}Cd, and the rate of Cd removal was determined. The perfusion solution was then replaced with glucose-saline containing 0.40 mM unlabelled Cd, a concentration adequate to severely depress fractional Cd transport. The tissue was allowed to accumulate Cd for 20 minutes before the perfusate was replaced with the original 0.02 mM Cd solution. Cadmium transport was now measured again and found to equal 51 percent (SD 29 percent) of the original control. In 4 further studies, the 0.4 mM Cd was omitted in period 2 in order to evaluate the influence of experimental procedures on the stability of Cd transport; in this case the mean activity in period 2 equalled 52 percent of that in period 1. Although these results were highly variable, they nevertheless suggest that the specific action of Cd in depressing, at higher concentrations, the fractional Cd absorption from the intestine, is largely reversible. Further evidence for reversibility was sought in 6 consecutive studies in which intestines were perfused at constant tracer concentration, first in presence of 0.02 mM Cd, then at 0.20 mM Cd, and finally again with the low Cd concentration. Results of these studies are collected in Table 2 and show that simple dilution can at least partially reverse the inhibitory effects of higher Cd concentrations.

DISCUSSION AND CONCLUSIONS

It is important to emphasize that results described in this paper do not refer to the transmural movement of Cd, but only to its transport from lumen into intestinal wall. The term *transport* is used advisedly, as simple physical diffusion cannot readily explain the saturation kinetics observed here. The rate of this transport is relatively fast, and it obviously does not limit transmural Cd uptake. Such a conclusion in turn implies that transfer of Cd into the body is determined by the rate of its release from the intestinal wall. Prolonged reten-

tion in the mucosal cells, as pointed out by others (e.g. Richards and Cousins, 1974) could lead to return of Cd to the lumen upon sloughing of cells. This fact presumably contributes to the generally low net absorption of Cd.

Characteristics of the process of Cd transport out of the lumen remain poorly defined. Under present conditions the process appears to obey saturation kinetics, with an approximate K_M of 0.1 - 0.2 mM (Fig. 4), and a V_{max} of the order of 0.01 μmol/g intestine/minute. Such a saturable process would contribute a major portion of total Cd absorption only at relatively low Cd concentrations, such as might be encountered under natural conditions. In contrast to present results, Kojima and Kiyozumi (1974) did not observe saturation kinetics and concluded that Cd outflux from the lumen obeys first order kinetics. These experiments were carried out at higher Cd concentrations than used here. Although our results provide no basis for such an assumption, it is possible that a second component of Cd translocation exists which does not become saturated in the concentration range studied. Evidence for two mechanisms of Cd uptake by duodenal tissue *in vitro* was also provided by Hamilton and Smith (1978). Koo *et al.*, (1978), in their work on transmural Cd movement out of the chicken intestine, studied Cd concentrations as high as 1 mM. Their conclusion that such Cd uptake could not be saturated is at variance with their observation of partial saturation over the concentration range of 0.01 to 0.10 mM. In addition, fractional Cd accumulation in duodenal tissue was clearly depressed by higher Cd concentrations. In any case, the saturable component studied here appears to be associated with mucosal cell membranes. This conclusion is based on the reversibility of saturation by simple dilution, a procedure which does not lead to significant washout of Cd from the tissue (Fig. 3). Work is in progress to determine whether the Cd accumulated in the tissue is concentrated in mucosal cells.

The great variability encountered in the kinetic analysis of Cd movement has so far precluded attempts to distinguish between competitive and non-competitive inhibition of Cd uptake by Ca. Calcium uptake by duodenal mucosa *in vitro* was reported to be non-competitively inhibited by Cd (Hamilton and Smith, 1978). Inversely, Ca exerted no effect on Cd uptake in this study, but, as in the work of Kojima and Kiyozumi quoted above, relatively very high Cd levels were employed.

Inhibition of Cd transport by milk illustrates the expected critical importance of the composition of luminal fluid. An influence of diet on Cd uptake into the body has often been reported, but it must be emphasized that changes in net, long-term Cd retention may not be related to acute effects of dietary constituents on unidirectional Cd flux into the gut wall. Thus, present results on the inhibition of Cd uptake by milk do not contradict the increased net Cd retention seen in young rats on a high milk diet by Kello and Kostial (1977).

Ability to transport Cd varies markedly along the jejunum (Fig. 1). Similar activity gradients for other solutes are well known. Further work will be necessary to explore the contributions of duodenum and ileum to total Cd transport.

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ACKNOWLEDGMENTS

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Paper B

Toxicology, 14 (1979) 199–208
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ON THE ROLE OF METALLOTHIONEIN IN CADMIUM ABSORPTION BY RAT JEJUNUM IN SITU

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SUMMARY

The rcle of metallothionein (MT) in the mechanism of cadmium absorption from the jejunum was studied in 7—9-week-old-male rats exposed to 50 ppm of cadmium in drinking water for 9 days. Exposed animals contained an average of 144 μg MT/g of mucosal tissue, compared to 40 μg in control animals. During jejunal perfusion in situ with 5 mM glucose-saline containing 10—20 nM $CdCl_2$ the increased MT content of mucosa exerted no effect either on cadmium absorption from the lumen (step I), or on its further transport into the body (step II). Immediately after perfusion, essentially all cadmium removed from the lumen was fully recovered in the intestinal mucosa. About 50% of the mucosal cadmium was found in the sediment after homogenization and centrifugation; a large portion of this cadmium may be assigned to the membrane fraction. The binding of freshly absorbed cadmium in the mucosal cytosol was not restricted to low molecular weight protein, although cadmium binding capacity in the MT fraction of controls as well as of exposed animals greatly exceeded actual binding of newly absorbed cadmium. Our results offer no support for the view that MT in the jejunal mucosa serves as determinant of cadmium absorption.

INTRODUCTION

Sufficient evidence has been accumulated in recent years suggesting that

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Abbreviation: MT, metallothionein.

oral intake represents the primary source of the cadmium accumulated in the human body [1—4]. Although cadmium absorption from the gastrointestinal tract has been investigated in a number of studies, the exact mechanism of cadmium transport from the intestinal lumen to blood is still unknown [3,5,6].

It has been suggested that MT, the low molecular weight Cd-binding protein found in mucosa [7—10], plays an active role in cadmium absorption from the gastrointestinal tract [6,11]. Although it is generally believed that intestinal MT is capable of sequestering intracellular cadmium, thus preventing transfer of the metal to the basolateral membrane, evidence for such a conclusion remains incomplete. It has even been proposed that the intestinal absorption of cadmium might possibly be increased in animals pretreated with cadmium [12].

The purpose of the present experiments was to study the role of mucosal MT in cadmium transport by rat intestine. Both uptake of cadmium from the lumen [13] (step I), and its further movement into the body (step II) were measured in animals as a function of intestinal MT levels. Results obtained permit the conclusion to be drawn that under present experimental conditions, MT does not influence Cd transport by rat jejunum.

MATERIALS AND METHODS

Animals and diets

Male rats of the Sprague—Dawley strain aged 7—9 weeks were obtained from Charles River Co. and maintained on Purina rat chow. This commercial diet contained an average of 0.11 ppm of cadmium, 65 ppm of zinc and 355 ppm of iron (supplier's analysis). Half the animals were exposed for 9 days to 50 ppm cadmium (as $CdCl_2$) in deionized water, offered ad lib; control animals received regular tap water.

Intestinal uptake and transport of cadmium

The rats were fasted for 24 h prior to the experiment. Anesthesia was induced by intraperitoneal injection of pentobarbital sodium (50 mg/kg). The abdomen was opened by a midline incision, and the proximal jejunum was identified, starting at the ligament of Treitz. A segment approx. 15 cm in length was selected and ligatures were loosely applied proximally and distally, care being taken not to compromise the mesenteric circulation to the bowel. Inflow and outflow catheters were inserted and sutures were tightened; the abdominal incision was closed with surgical clamps. Body temperature was maintained with a heating pad and was monitored with a rectal thermometer. The lumen of the jejunal segment was perfused with 10 ml of solution (saline with 5 mM glucose) containing 200 nmol cadmium chloride labeled with 0.5 μCi ^{109}Cd (New England Nuclear, Boston, MA), at a flow rate of 0.4 ml/min for 25 min. The concentration of Cd of 0.02 mM is lower than that used by many other investigators. It was chosen because it does not saturate the mechanism responsible for Cd transport at levels which might be encountered in polluted environments [13]. Immediately

following the perfusion, the lumen was rapidly flushed with 10 ml of ice-cold 0.15 M NaCl followed by 10 ml of air. Blood (6—8 ml) was collected from the abdominal aorta. The jejunal segment, liver and kidneys were removed, weighed and their radioactivity counted in an automatic well-type scintillation counter (Packard, Model A-5921). Mucosa was scraped from the serosal layers of the entire length of perfused jejunal segment with a glass slide, weighed, counted, and stored at −65°C. The ^{109}Cd activity of the remaining carcass of each rat was determined in a whole-body counter using a 5 × 5 inch NaI (TL) crystal connected to a Packard analyzer (Packard, Model A-5921). Results are expressed as nmol $CdCl_2$, as calculated from the specific activity of the solution.

Rate of transmural transport of cadmium

Because the amount of Cd taken up by the jejunum very greatly exceeds that further transported into the body, i.e. because the rate of step I greatly exceeds that of step II, accurate comparison of the 2 steps and an appropriate mass-balance of transported Cd could not be readily achieved with the whole body counter. A technique was therefore developed [14] for measurement of steps I and II on the basis of mass-balance measurements. In a manner similar to that previously described [13], a small volume (1.6—2 ml) of the same solution as above was recirculated through the jejunal lumen at 0.8 ml/min; perfusion pressure did not exceed 2 cm H_2O. The rate of step I was computed from the fall in the ratio of $^{109}Cd/^{3}H$, as determined with a Packard liquid scintillation spectrometer. At the end of perfusion remaining perfusate was collected and combined with 3 ml saline used to wash out the lumen. Quantity A, the amount of Cd removed from the lumen, was calculated from the total ^{109}Cd content of this solution, as measured on a Packard well-type scintillation spectrometer. If, immediately after washing, the intestine was cut out and its ^{109}Cd content (quantity B) determined, essentially all Cd removed from the lumen could be recovered in the tissue (Fig. 1). To determine step II, the catheters were clamped and the abdominal incision closed. Two hundred and seventy-five minutes later, the segment of jejunum was removed for determination of its radioactivity, together with that of its contents. At this time, recovery from the tissue fell below the amount originally absorbed by the tissue, i.e. $A > B$. The deficit in recovery (C), where $A - B = C$, provides a measure of step II. After it had been blotted on the outside, the tissue was then weighed, so that the rates of steps I and II could be calculated in nanomoles/g fresh wt/min.

Cadmium binding components in perfused jejunal mucosa

To determine which tissue components react with freshly absorbed cadmium, mucosa obtained from perfused jejunal segments was homogenized in 0.25 M sucrose (1 : 4 w/v) and centrifuged at 100 000 *g* for 1 h. The radioactivity in supernatant and precipitate was determined. The supernatant was chromatographed on a Sephadex G-75 column (1.5 × 30 cm) in 20 mM Tris—HCl buffer, pH 8.6 and the radioactivity of each fraction was

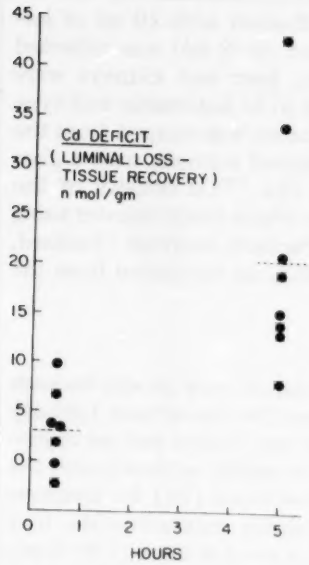

Fig. 1. Quantitation of step II by measurement of the difference (C) between Cd removed from the lumen during 25 min perfusion (A) and that recovered from the intestinal wall at the times shown (B), i.e. C = A − B. Time is measured from the beginning of perfusion, and mean deficits are indicated by dashed lines.

measured. The results are expressed as percent of total ^{109}Cd radioactivity in homogenate.

Determination of intestinal metallothionein

The total amount of MT in the mucosa was determined with the saturation method described by Kotsonis and Klaassen [15]. Homogenized mucosa (25% in 0.25 M sucrose) was centrifuged at 10 000 *g* for 15 min, 10% TCA was added to the supernatant to a final pH of 2. The mixture was centrifuged again at 15 000 *g* for 30 min. The TCA supernatant was mixed with cadmium solution (300 nmol $^{109}CdCl_2$/g tissue). The pH was adjusted to 8.6 with dilute NaOH and the solution was fractionated on a Sephadex G-75 column (1.5 × 30 cm) and the radioactivity of each fraction was determined. The amount of cadmium in the MT peak was calculated from the specific activity of the $^{109}CdCl_2$. The amount of Cd-binding protein of low molecular weight was then derived from its Cd-binding capacity on the assumption that it binds 7 mol Cd/mol of 10 000.

Determination of cadmium and zinc

The intestine was thoroughly washed with saline before the analysis. Cadmium and zinc were determined in mucosa by atomic absorption spectro-

photometry (Perkin Elmer, Model 403) after wet digestion with nitric and perchloric acid. In samples from control group of animals cadmium was extracted and concentrated using the method described by Yeager et al. [16].

Statistics

All results are expressed as the arithmetic mean with the standard deviation of the mean. The significance of the difference among the groups was determined by Student's *t*-test for unpaired data.

RESULTS

Induction of metallothionein in intestinal mucosa

The daily intake of cadmium in animals exposed to 50 ppm cadmium in drinking water has been estimated as 1.4 mg, i.e. total intake of cadmium after 9 days was about 12.6 mg. Data presented in Table I show that such treatment caused an increase in the amount of cadmium and metallothionein in mucosa. Mucosal cadmium in pretreated rats was 68 times higher than in control animals; at the same time the concentration of metallothionein increased only 3.6 times. The concentration of zinc in these 2 groups of animals did not differ significantly.

Intestinal uptake and transport of cadmium

The data presented in Table II demonstrate that after intestinal perfusion with 0.02 mM $^{109}CdCl_2$ solution for 25 min, extensive accumulation of cadmium was found only in the intestinal wall. By contrast, the total intestinal transfer of cadmium, expressed as the sum of cadmium recovered in carcass, liver, kidneys and blood, represented less than 0.3 percent of the total perfused dose in both groups of animals. As further described below essentially all cadmium accumulated by the intestine was confined to the mucosa.

TABLE I

CONCENTRATIONS OF CADMIUM, ZINC AND METALLOTHIONEIN IN MUCOSA (μg/g wet wt)

Results are expressed as arithmetic mean ± S.D. Exposed animals had 50 ppm of cadmium in drinking water for 9 days.

	6 Control rats	6 Pretreated rats
Cadmium	0.06 ± 0.04	4.32 ± 0.86
Zinc	21.5 ± 3.4	22.2 ± 4.1
Cd-binding capacity[a]	3.2 ± 0.5	11.3 ± 2.8
Metallothionein	40 ± 6	144 ± 36

[a]Equivalent to total amount of Cd bound in MT fraction, as measured during MT assay.

Although these results (Table II) show that the intestinal uptake of cadmium was slightly higher in pretreated than in control animals (17% and 13% of dose respectively, $P < 0.05$), the total transmural transport was the same in both groups.

Cadmium binding components in perfused jejunal mucosa

In order to determine the distribution of cadmium in the intestinal wall, the mucosa of perfused jejunal segments was harvested by scraping and analyzed. The supernatant fraction of mucosal homogenates in both groups of animals contained about one-half of total tissue ^{109}Cd (Table III). Although the supernatant from exposed animals contained a little more cadmium than that from controls, major differences between these 2 groups appeared after chromatography. As shown in Table III there were 2 major cadmium peaks in both groups. The second peak corresponds to a mol. wt of about 10 000; the first peak contains high molecular weight compounds. In control rats most of the ^{109}Cd appeared near the void volume, indicating that most of the cadmium was bound to high molecular weight proteins and very little to MT. In contrast, in the pretreated rats little cadmium was bound to high molecular weight proteins and more was associated with the low molecular weight fraction, tentatively identified as MT. In neither group did significant amounts of cadmium remain unbound.

Rate of transmural transport of cadmium

Because of the small amount of Cd transferred to the body during 25 min perfusion (Table II), the transfer step (step II) was measured over a period of 5 h as discussed in the Materials and Methods section. Table IV, based on results similar to those shown in Fig. 1, demonstrates that the rate of trans-

TABLE II

INTESTINAL UPTAKE AND TRANSMURAL TRANSPORT OF CADMIUM

Perfusate contained 200 nmol $CdCl_2$. Results are expressed as nmoles of cadmium (Mean ± S.D.). Rats were killed for tissue analyses after 25 min perfusion.

	15 Control rats	15 Pretreated rats
Weight of intestine (g)	1.4 ± 0.3	1.4 ± 0.2
Intestinal uptake	25.1 ± 6.5	34.0 ± 13.3
Retained in mucosa	24.5 ± 6.4	33.1 ± 12.8
Carcass	0.211 ± 0.091	0.277 ± 0.192
Liver	0.186 ± 0.108	0.252 ± 0.180
Kidney	0.007 ± 0.003	0.012 ± 0.006
Blood	0.007 ± 0.003	0.016 ± 0.009
Total transfer to body	0.412 ± 0.136	0.555 ± 0.319

TABLE III

DISTRIBUTION OF PERFUSED CADMIUM IN JEJUNAL MUCOSA

Results are expressed as percent of levels in original homogenate.

	5 Control rats	5 Pretreated rats
Homogenate	100.0	100.0
Sediment	51.4 ± 2.5	40.4 ± 5.7
Supernatant	48.6 ± 2.5	59.6 ± 5.7[a]
High mol. wt fraction (above 10 000)	30.0 ± 3.7	17.2 ± 1.8[b]
Low mol. wt fraction (approx. 10 000)	13.4 ± 5.1	38.9 ± 7.2[b]
Remaining fraction (below 10 000)	5.6 ± 1.0	3.4 ± 0.5

[a] $P < 0.01$.
[b] $P < 0.001$.

mural transport as measured by disappearance of cadmium over a period of 5 h was low and did not exceed 1.3% of the rate of cadmium removal from the lumen. The ratio of step II to step I was the same in both groups of animals.

DISCUSSION

While relatively little is known about the mechanism of cadmium absorption, it is clear that this process must consist of at least 2 steps: (1) uptake

TABLE IV

RATE OF UPTAKE AND TRANSMURAL TRANSPORT OF CADMIUM

Results are shown as mean ± S.D.

	10 Control rats	13 Pretreated rats
Intestinal uptake[a] (nmol/g/min)	1.9 ± 0.8	1.3 ± 0.4
Transmural transport[b] (nmol/g/min)	0.023 ± 0.010	0.015 ± 0.009
Rate of transmural transport as % of rate of uptake	1.3 ± 0.7	1.3 ± 0.8

[a] Jejunal segment was perfused with 0.02 mM $^{109}CdCl_2$ solution for 25 min.
[b] Rate of transport was calculated from difference between initial uptake and final retention of ^{109}Cd in tissue 275 min after the end of perfusion.

of the metal by the mucosal cells; and (2) transmural movement of cadmium into the body [6,14,17]. Despite differences in methodology and species, there is considerable agreement between the results presented here and those obtained by previous workers [5,6,17], namely that intestinal uptake of cadmium is much higher than its transport into the body (Table II). Thus, we found that even after 5 h, only a small portion of cadmium accumulated in jejunal mucosa is released into the animal; the rate of release amounted to only 1–2% of the rate of uptake (Table IV).

It is important to emphasize that under present conditions, in the absence of normal luminal contents, uptake of cadmium in all likelihood greatly exceeds that to be expected in presence of normal intestinal contents. We may assume that in rats on a normal diet most of the metal is bound to non-absorbable food components and is therefore eliminated from the body [18]; there is evidence also for the presence in food of direct inhibitors of cadmium absorption [3].

The prolonged retention of cadmium by the intestinal tract was attributed to extracellular adsorption [17,19] or intracellular accumulation and binding on different ligands, particularly MTs [12,20]. Sahagian et al. [17] proposed that in the process of mucosal uptake, cadmium probably reacts with the cell membrane. This is fully compatible with the present study which shows that about one-half of ^{109}Cd present in mucosal homogenate is collected in the precipitate after centrifugation. Since numerous studies have shown that only a few percent of intracellular cadmium are bound to nuclei, mitochondria and endoplasmic reticulum [21], a large portion of the cadmium in the precipitate may be assigned to the membrane fraction. Similar findings by Taguchi and Suzuki [22] support this conclusion. Detailed information about the nature of binding sites or classes of ligands present in membranes for a given metal is not available.

Recently several authors suggested that intestinal MT plays an important role in cadmium absorption from the gastrointestinal tract [6,10,11]. It was postulated that this protein would sequester intracellular cadmium and prevent in this way its transport into the circulation [12,20]. This statement is based on the assumption that the sequestering of cadmium in the mucosa resembles the handling of iron [20] which, when taken up by the mucosa in excess of bodily needs, is believed to bind to ferritin and subsequently to be excreted upon desquamation of the epithelium. This hypothesis, though attractive, is not fully supported by available evidence. Thus, Sasser and Jarboe [19] and Foulkes and Stemmer (unpublished observation) observed accumulation of cadmium in the deeper mucosal layers which do not readily participate in the rapid turnover of epithelial cells. In addition, the binding of cadmium observed in the present study was pronounced even in control animals, although these contained only small levels of MT in the mucosa. Clearly, binding of cadmium is not restricted to MT, a conclusion which raises questions about the proposed primary role of this compound in the sequestration of the metal.

A similar conclusion can be drawn from the fact that the great increase

in MT seen in exposed animals was not accompanied by either significant increases in cadmium uptake or increases in its retention in the mucosa. Indeed, as can be deduced from Table I, an increase in the Cd-binding capacity of the MT fraction from 28 to 100 nmol/g mucosa exerted no effect on the transmural transport of cadmium (Tables II and IV). Even a partial involvement of the relatively very large Cd-binding capacity in the mucosal MT fraction should have been reflected in some change in the very much smaller amounts of cadmium transported across the tissue. The small increase in accumulation of ^{109}Cd in mucosa of pretreated rats (Table II) may perhaps be explained by alterations in the permeability of the membranes [20,23].

Different authors found that cadmium in the cytosol of intestinal tissue is mainly bound to MT [7—10]. We observed that cadmium, freshly absorbed by control animals, reacts primarily with high molecular weight proteins rather than with MT (Table III), although the mucosa actually contained significant amounts of the protein (Table I). Presence of MT in normal animals has previously been reported by other authors [8,20], and presumably reflects normal background levels induced by zinc in the diet. Our calculations show that cadmium binding capacity of MT, even in control animals (Table I), greatly exceeds actual binding of newly absorbed cadmium (Tables II and III). Kotsonis and Klaassen [11] recently reported similar findings in experiments involving measurement of Cd-binding capacity of MT in liver and kidneys. Apparently, even though MT may not be fully saturated with metal, a variety of ligands can and do compete for freshly absorbed cadmium. Obviously, a short segment of jejunum in the surgically prepared animal is not fully representative of the intestine in an intact rat. Nevertheless, our results clearly show that MT in the mucosa does not serve as a primary determinant of cadmium absorption, at least under present experimental conditions.

ACKNOWLEDGEMENTS

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Paper C

(Submission Draft)

RELATIONSHIP BETWEEN CADMIUM AND ZINC ABSORPTION BY RAT JEJUNUM

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SUMMARY

Interaction between Cd and Zn during their absorption from the intestinal lumen was studied in the rat jejunum perfused in situ. Cadmium and Zn depress each other's transport out of the lumen in an apparently competitive manner. The two metals presumably share, in part, a common absorption mechanism. However, important quantitative differences are seen in the second step of absorption, i.e. transfer from mucosa into the body. Overall absorption of Cd and Zn may thus be mediated by differing mechanisms.

INTRODUCTION

Interaction between Cd and Zn has been extensively documented in many tissues (1) including the intestine (2-4). However, the mechanism of absorption of heavy metals is complex, and the nature of Cd-Zn interaction is not understood. The complexity derives in part from the fact that more than one step is clearly involved in heavy metal absorption. The first of these (step I) represents uptake of metals from lumen into the mucosa, while step II consists of their further transfer into the body (5). The observation that Zn depresses step I of Cd transport (2) raises the question to what extent these two metals share a common absorption mechanism. It is the purpose of the present paper to explore this question.

MATERIALS AND METHODS

Male Sprague Dawley strain rats aged 7-9 weeks were obtained from Charles River Co., and maintained on Purina rat chow. The rats were fasted for 24 hours prior to the experiment. Anesthesia and surgical procedures have been previously described in detail (2, 5). Briefly, the lumen of a segment of proximal jejunum

approximately 12 cm in length was perfused in situ. The perfusate consisted of 2.4 ml saline solution containing 5 mM glucose and was recirculated at a rate of 0.8 ml/min.

For measuring uptake of Cd, cadmium chloride labelled with ^{109}Cd (0.13 µCi) and 0.63 µCi ^{3}H-polyethylene glycol as volume marker were added to the recirculating system. Disappearance of Zn from the lumen was determined in presence of $ZnCl_2$ labelled with ^{65}Zn (0.625 µCi) and 0.02 µCi ^{14}C-polyethylene glycol. Transport of Cd and Zn from the lumen was calculated from changes in $^{109}Cd/^{3}H$ and $^{65}Zn/^{14}C$ ratios as determined on a Packard liquid scintillation spectrometer with automatic external standards.

Measurement of step II in Zn absorption followed essentially the technique previously described for Cd (5). Jejunum was perfused with 0.05 mM Zn solution for 20 min. At that time, final perfusate and washings were collected to determine on a gamma counter the amount of ^{65}Zn removed by the intestine. This amount was compared with the ^{65}Zn retained in the intestinal wall. As in the case of Cd, a deficit in tissue recovery was equated to transfer from tissue into body, i.e. step II.

RESULTS

As previously shown (2), Zn inhibits step I of Cd absorption. This is confirmed by the lines of best fit in Figure 1 which reports results from studies at three different Cd concentrations in presence of three inhibitory levels of Zn. The large variance in the results reflects the previously noted variance between animals (2). In spite of this, the figure shows that with increasing concentration, Zn increases the slope of the double reciprocal lines without significantly altering their small intercept on the ordinate. Such

a result is compatible with competition between Cd and Zn. Figure 2 shows similarly that Cd inhibits Zn transport, again in a manner suggesting competitive interaction.

Table I summarizes experiments on the rates of steps I and II of Zn transport. Unlike the essentially full recovery in the tissue observed for Cd after 25 min perfusion (5), a large fraction of the Zn removed from the lumen in 20 min was transported further during that period. In 10 studies, a mean recovery of ^{65}Zn in tissue of only 36 ± 7% of that removed from the lumen could be obtained, i.e. the rate of step II of Zn transport approximates 64% of that of step I; the corresponding value for Cd, in contrast, is only 1-2% (5). A relatively rapid movement of Zn from gut into body has repeatedly been reported in the past (6, 8).

DISCUSSION

The finding that Cd and Zn may compete with one another at the level of step I in their absorption suggests that they share, at least in part, a common uptake mechanism, and that a common binding site may be involved in their transport. It is worth recalling that common mechanisms for Cd and Zn transport have been previously suggested (7).

While a common mechanism can therefore explain transport of Cd and Zn out of the lumen, their further movement into the body (step II) is quantitatively very different. Thus, Cd is tightly bound in the mucosa (3), whereas Zn rapidly enters the body (see Table I). The difference in step II indicates that the overall absorption processes of the two metals are not identical.

The question whether metallothionein is involved in the common pathway of Cd and Zn transport can be answered in the

negative on the basis of our earlier results (5). Similarly, if low molecular weight ligands are involved in Zn transport, then the inability of Cd to react with such a compound, as reported by Sugawara, et al. (9), suggests that the ligands also cannot form a common element in the uptakes of Cd and Zn.

ACKNOWLEDGMENTS

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TABLE I

STEPS I AND II IN ZN TRANSPORT

	% of initial Zn content in perfusate (Mean ± SD)
Removed from perfusate (step I)	52.3 ± 7.4
Recovered from intestinal wall	18.8 ± 2.4
Deficit (step II)	33.5 ± 7.4
Step II/I (%)	64.0 ± 7.0

Data were obtained from 10 rats. Each jejunum was perfused for 20 minutes with 2.4 ml perfusate containing 120 nmoles Zn.

Figure 1. Effect of Zn on Cd uptake. Cd was transported out of the lumen at a constant exponential rate for 30 min, from which the mean initial rate (M ± SE) of transport was calculated in nmole Cd/g tissue/min for 4 to 6 rats.

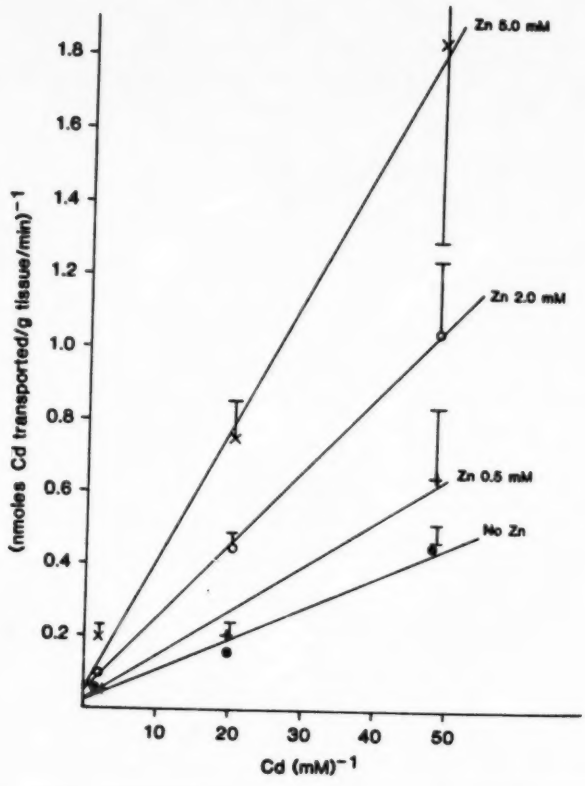
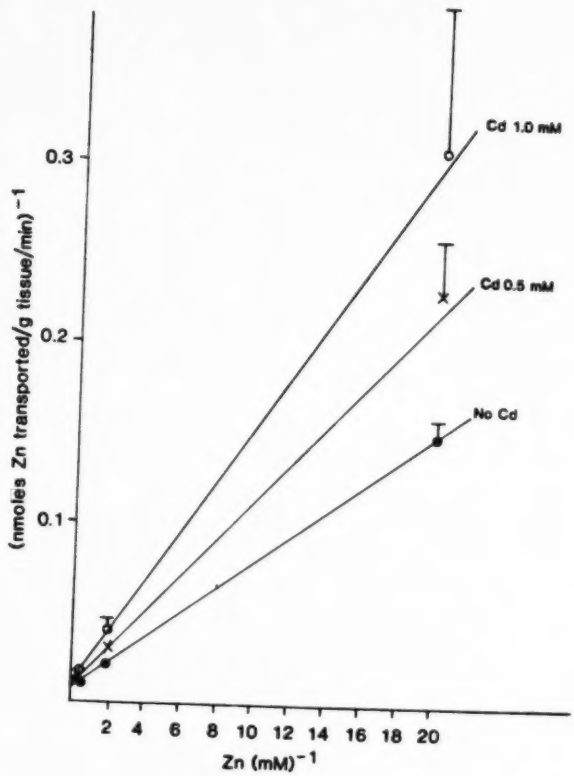


Figure 2. Effect of Cd on Zn uptake. Zn was transported out of the lumen at a constant exponential rate for 6 min, from which the mean initial rate (M ± SE) of transport was calculated in nmole Zn/g tissue/min for 3 to 10 rats.



Abstract A

PHYSIOLOGY

STEPS IN CADMIUM TRANSPORT BY RAT JEJUNUM IN SITU. E.C. Foulkes and C. Voner, Depts. Environ. Health & Physiol., Univ. of Cincinnati Med. Center, Cincinnati, Oh. 45267.

The fact has been repeatedly observed that uptake of Cd from the intestinal lumen (step #1 of Cd transport) is much faster than its further movement into the body (step #2). Less well documented is a quantitative comparison of steps 1 and 2 *in vivo*, at low Cd concentrations approaching those which might be encountered in dietary exposure. The mechanism mediating step #1 at these low levels becomes saturated at the higher concentrations used in much of the earlier work (J. Env. Path. Toxicol., in press). At a concentration in luminal perfusate of 20 μM Cd, step #1 proceeds at 2.0 ± 0.7 (SD, n = 17) nmoles/g fresh weight jejunum/min. Immediately after 25 minutes' perfusion essentially all Cd removed from the lumen could be recovered from the intestinal mucosa. If 5 hours were allowed to elapse before excision of the intestine, a significant fraction of Cd taken up during perfusion had moved beyond the intestine. This deficit may be attributed to step #2, and was incurred at a mean rate of 0.03 ± .02 nmol/g/min. The ratio of steps 2/1 equalled 1.7 ± 1.0%. We have shown elsewhere that the long retention of Cd in the intestine is not a function of endogenous metallothionein levels (Toxicol., in press). These could be raised by 360% above control levels during exposure to 0.5 mM Cd in drinking water, without significantly altering steps 1 or II. The factors determining Cd retention remain undefined. (Supported by EPA grant R-805840-010).

Abstract B

MODIFICATION OF JEJUNAL Zn AND Cd TRANSPORT IN RATS BY GLUCOCORTICOID NOT CORRELATED WITH METALLOTHIONEIN SYNTHESIS. R.F. Bonewitz and E.C. Foulkes (Spon. P.B. Hammond) Dept. Env. Health, Univ. Cincinnati Med. Ctr., Cincinnati, OH 45267.

Hepatic and intestinal metabolism of Zn, Cd, and several other heavy metals may involve metallothionein (MT). Synthesis of MT is induced in many tissues by these metals. Furthermore, glucocorticoids have been found to induce MT synthesis in some cultured cells and in liver of adrenalectomized (ADX) rats. We investigated effects of dexamethasone (dex) (2.0 mg/kg i.p.) on removal of ^{65}Zn (20-100 µM in 5 mM glucose-saline) from the lumen of jejunal segments perfused *in situ*, and on induction of mucosal MT. In controls not given dex, uptake was first order ($k = 2.18 \pm 0.53 \times 10^{-2}$ $min^{-1}gm^{-1}$, 95% C.I.). In contrast, in animals given dex 7 h (but not 0, 1, 4, or 12 h) before assay, uptake was biphasic with a rapid component ($k \geq 6.84 \pm 0.84 \times 10^{-2}$ min^{-1} gm^{-1}) which was completed in ≤3 min, and a slower component not significantly different from controls. Results were not affected by adrenalectomy. Dex had a qualitatively similar effect on first-order ^{109}Cd uptake. Dex and ^{35}S-cystine (15.9 µCi, i.p.) were administered 7 and 3 h, respectively, prior to sacrifice, and mucosal cell cytosol was chromatographed on Sephadex G-75. In neither dex-treated nor untreated animals was significant ^{35}S incorporated into MT. Control studies confirmed ^{35}S-labelled MT synthesis in mucosa of Zn^{2+}-TREATED (150 µmol Zn^{2+}/kg, i.p.) rats and in liver of dex-treated ADX rats. The results indicate that dex-modifies intestinal Zn transport, apparently by creating a second compartment, possibly a sink, accessible to luminal Zn^{2+}. The results further show that MT is not detectably induced by dex and is therefore unlikely to be involved in this transport effect.

Abstract C

PHYSIOLOGY

INHIBITION OF JEJUNAL Cd ABSORPTION IN THE RAT BY BILE SALTS. C. Voner* and E.C. Foulkes, Depts. of Env. Health & Physiol., Univ. of Cincinnati Med. Cen., Cincinnati, OH 45267.

Composition of food is well-known to influence intestinal absorption of various heavy metals; in addition, endogenous factors also may play a role. We report here effects of bile on removal of Cd from the lumen of the proximal jejunum of the rat perfused *in situ*. In these experiments 1.6 ml saline, containing 5 mM glucose, 20 µM $CdCl_2$ labelled with 0.15 µCi ^{109}Cd, and 0.75 µCi ^{3}H-polyethyleneglycol as volume marker, were recirculated at 0.8 ml/min through a 10 cm length of jejunum. Cd absorption was inhibited by secretions collected from the duodenum, but no inhibition was seen after ligation of the common bile duct. Addition of 20% (v/v) fresh rat bile reversibly decreased Cd uptake from 5.8 ± 1.6 to 3.2 ± 1.1%/g /min (SD, n=6). This action could be duplicated with glycocholic (GC) or taurocholic acids. Above 6-9 mg/ml GC abolished Cd transport; below 5 mg/ml, little inhibition was noted. It may be significant that the critical micellar concentration of GC lies in the range of 4-8 mg/ml. Bile salts can clearly serve as physiological modulators of Cd absorption. (Supported in part by EPA grant R-805840010 and NIH grant ES-00159)

Abstract D

PHYSIOLOGY

INTESTINAL TRANSPORT OF CADMIUM IN NEWBORN RATS. D.R. Johnson, E.C. Foulkes and L. Leon*, Depts. Env. Health and Physiol., Univ. Cincinnati Med. Cen., Cincinnati, OH 45267.

Considerable attention has been given to absorption of Cd from the small intestine of adult rats. However, absorption of metals, including Cd, is greater in newborn rats than adults. The present studies were conducted to investigate intestinal Cd transport in the newborn rat. Transport *in situ* from duodenum and jejunum of 14 day old rats was measured thirty minutes after placing 0.1 ml 20 µM $CdCl_2$ labelled with 0.02µCi ^{109}Cd into the intestinal lumen. Duodenal uptake from lumen to mucosal cell and absorption from cell to blood were 2.4 ± 0.6%/min/0.1g and 0.5 ± 0.4%/min/0.1g (S.D., n=5), respectively. Uptake and absorption from jejunum were 1.0±0.3 and 0.20 ± 0.05%/min/0.1g, respectively. Although duodenal transport was greater than jejunal, the absorption to uptake ratio in these two segments was equal. Both rate of transport and ratio of absorption to uptake are greater in the newborn than in the adult. Unlike in adults, neither chronic nor acute administration of iron to pups altered Cd uptake from jejunum. Cd uptake in the presence of 200 µM $FeSO_4$ was 0.9 ± 0.2%/min/0.1g. Feeding of milk supplemented with 200 µM $FeSO_4$ for 7 days (day 7-14) did not alter the rate of Cd uptake even though mucosal iron level was doubled. These results suggest that the mechanism of intestinal Cd transport in the newborn rat differs quantitatively and qualitatively from that in the adult. (Supported in part by EPA grant R-805840010 and NIH grant ES-00159)

Abstract E

COMPARISON OF MUCOSAL UPTAKE AND TRANSMURAL TRANSPORT OF Zn, Cu, AND Cd IN RAT JEJUNUM. Roland F. Bonewitz, Jr.*, Cathleen Voner* and E.C. Foulkes, Depts. Env. Health & Physiol., Univ. Cincinnati Med. Ctr., Cincinnati, OH 45267.

Intestinal absorption is a major route for the assimilation of both nutritionally essential and toxic metals by the body. The low net fractional absorption of Cd suggests however that the intestine may possess a mechanism for discriminating between Cd and essential metals. Mucosal (M) uptake and transmural transport of Zn, Cu, and Cd were compared under identical conditions in segments of the adult rat jejunum in situ. 20 μM metal salt + tracer in 5mM glucose-0.15M NaCl was recirculated through the lumen (L). L→M transfer (Step I) of all metals was 1-2 nmol $min^{-1}g^{-1}$. However, the further transport into the body (Step II) was higher for Zn and Cu (48% and 47%, respectively, of Step I) than for Cd (<2%). Discrimination against Cd thus occurs past the level of M uptake. As the segment was isolated and perfused with a medium lacking added ligands, and as tissue through which perfusate was not recirculated suffered no decrease in the rate of Step I, it is unlikely that specific metal binding ligands accumulated in L to mediate metal absorption. Although some ligands present in food or secreted into the intestine may enhance metal absorption through competition with food constituents for metal binding, such ligands are apparently not specific and obligatory components of the uptake mechanism. (Supported by NIH grants ES00159 and ES07073 and EPA grant R805840)